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14. ABSTRACT
Prostate cancer is the most common non-cutaneous malignancy and second most common cause of cancer-related deaths in United States men. Despite extensive research efforts the molecular pathogenesis responsible for prostate cancer development and progression is not well understood. Previously most studies have been genomic based and have focused on discovery alterations in DNA or RNA expression. The emerging field of proteomics has provided researchers the opportunity to study proteins and protein modifications that are directly responsible for the molecular phenotype. Recently, it was discovered that one protein, named Nedd8, is under expressed in prostate malignant tissues, which could result in the loss of neddylation of key proteins, such as cullin family members. This modification is very important to ensure the proper function of cullin proteins since they are involved in degradation of many critical regulator proteins through proteasome pathway to maintain proper cell growth. Abruption in these pathways will cause accumulation of unwanted proteins and prevent cells from normal growth. Thus it will result in protein malfunction in our body and promote cancer development. Therefore, better understanding of Nedd8 pathway in human prostate cancer would provide a direct link between protein degradation pathways and prostate cancer development and tumorigenesis. The idea in this proposal is new and innovative since this line of research has not been tackled before. Any finding in this proposal will help to elucidate the biological role of Nedd8 pathway in human prostate cancer development. The improved understanding in the development of prostate cancer due to Nedd8 pathway may thus providing an important new target platform for future mechanism-driven drug discovery for treatment of prostate cancers, and maybe other cancer as well.

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Introduction

Recently proteomic analysis has discovered that Nedd8 (neutral precursor cell expressed and developmentally down-regulated 8) expression level is dramatically reduced in human prostate cancer compared to benign prostatic epithelium. In addition, a number of high molecular weight Nedd8 modified proteins were observed, and one of them was markedly reduced in intensity (2-fold) in the malignant tissue. The results suggest that under-expression of Nedd8 will result in forming of less number and/or less amount of Nedd8 modified proteins in prostate cancer. Our hypothesis is that Nedd8 regulates the balance between cell proliferation and cell death by controlling the formation of specific cullin-containing ubiquitin ligase through neddylation and that loss of Nedd8 leads to reduced degradation of proliferative and anti-appoptotic proteins. Novel targets for neddylation in prostate tissue could identify specific mediators of prostate cancer development and progression. Therefore, the objective of this project is to study Nedd8 pathway in human prostate cancer employing mass spectrometry-based approaches to identify and characterize Nedd8 modified proteins from human prostate tissues. An additional objective is to establish a model system using benign and malignant prostatic epithelial cells for future study of the biological role of Nedd8 pathway associated with down-regulation of Nedd8 expression in prostate cancer.

To achieve these goals, we have spent most of our time during last year to carry out experiments for tagging Nedd8 at N-terminus and affinity purification of the Nedd8 modified proteins and their associated proteins. Mass spectrometry-based proteomic approaches have been employed for comprehensive identification and characterization of Nedd8 modified proteins from cell lines expressing tagged Nedd8. Our preliminary results are very exciting and promising, which provide a strong basis for us to further our analysis as proposed in this work. The detailed process report is described below.

Body

Task 1*. Identify Nedd8 modified proteins in human benign prostate tissues from radical cyto-prostatectomy specimens, Months 1-12:*

a. Isolate Nedd8 modified proteins by affinity purification using anti-Nedd8 specific antibodies, separate by 2-D electrophoresis and distinguish Nedd8 modified proteins from others using western blotting.
b. Protein identification using mass spectrometry.
c. Characterize neddylation by mass spectrometry.

In order to carry out the proposed experiments, we have first tested four different anti-Nedd8 specific antibodies from three different companies (Biomol, PA; Alexis Biochemicals, CA; Santa Cruz Biotechnology, Inc., CA). for both immunoblotting and immunoprecipitation experiments. The experimental results revealed that all the commercially available antibodies are somewhat suitable for immunoblotting with different sensitivity and specificity; however, none of them are usable for immunoprecipitation purposes. Therefore, we contacted an antibody company (Genetex, Inc.) at Texas to raise polyclonal anti-Nedd8 antibody for the proposed research at the end of year 2004. Due to the unavailability of the anti-Nedd8 antibody, we have postponed this part of proposed work.

Task 2. *Establish cell model system for study of biological impact of Nedd8 pathway in human prostate cancer---Determine expression of free Nedd8 and Nedd8 modified proteins in benign and malignant prostate epithelial cells (PrEC and LNCaP), Months 13-29:*

a. Determine expression of free Nedd8 and unknown targets by neddylation in these two cell types using 2-D electrophoresis, western blot and mass spectrometry.

Due to the specificity and sensitivity of the commercially available Nedd8 anitbodies as addressed above, we have decided to postpone this proposed work and wait until the specific antibody is available.

b. Affinity purify Nedd8 modified proteins using FLAG at N-terminus of Nedd8.

We have first started this line of work by tagging 3xFLAG at N-terminus of Nedd8 to affinity purify Nedd8 modified proteins. The pET3a-Nedd8 plasmid (kindly provided by Prof. Cecile Pickart at John Hopkins University) was inserted into the BamHI site of mammalian expression plasmid p3XFlag-CMV7 (Sigma) to generate p3xFlag-Nedd8. In our first attempt, Hela cells were chosen and transiently transfected with p3XFLAG-Nedd8 using Lipofectamine™ 2000 (invetrogen) according to the manufacturer's protocol. After 24 hours transfection, the cells were lysed first in Buffer A (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 0.5% NP-40, 1 mM PMSF) and then in Buffer B (20 mM Tris-HCl pH 7.4, 1 M NaCl, 0.2% NP-40, 1 mM PMSF) with the help of the passage through 20G needle for 20 times. The Mixture was agitated for 30 min at 4°C followed by centrifugation at full speed at 13,000 rpm for 30 min. Supernatants were extracted for affinity purification of p3xFLAG-Nedd8 modified proteins using anti-FLAG M2 Affinity Gel (Sigma) based on the manufacturer's protocol. Briefly, after overnight binding at 4°C, the p3xFLAG-Nedd8 modified proteins bound on the anti-FLAG M2 affinity gel were eluted by 100 ul of 100ug/ml of 3xFLAG Peptide. To evaluate the p3xFLAG-Nedd8 modified proteins and purification efficiency, we have subjected the cell lysate and affinity purified protein fractions for 1-D SDS-PAGE and subsequently western blotting using anti-FLAG antibody as shown in Fig.1. Wild type Hela cells without transfection was used as a negative control. Fig. 1A displayed the western blot of the cell lysates from both non-transfected and transfected, whereas Fig 1B illustrates the western blot from the eluted fractions after affinity purification as described above. Clearly, in comparison to the control, both the lysate and elute from the cells transiently expressing p3XFLAG-Nedd8 have shown many proteins

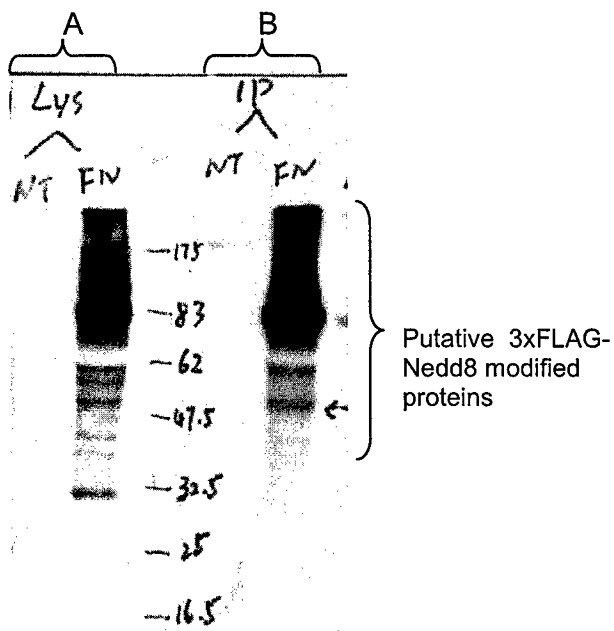

Fig.1 Detection of p3xFLAG-Nedd8 modified proteins by immunoblotting using anti-FLAG specific antibody .(A), cell lysates from Hela cells expressing p3XFLAG-Nedd8; (B), affinity purified fraction from (A). NT—non-transfected cells, i.e. control cells; FN—p3XFLAG-Nedd8 transfected cells; Lys—lysate; IP—immnuprecipitation.

that are potentially neddylated based on the immunoblotting using anti-FLAG. In order to identify the putative Nedd8 modified proteins, we TCA precipitated the elute and used 8M urea/50mM NH_4HCO_3 dissolved the protein pellets and digested with endopeptidase Lys-C and subsequently with trypsin. The digests were analyzed by liquid chromatography on-line coupled with tandem mass spectrometry (LC MS/MS).

Briefly, LC MS/MS was carried out by nanoflow reverse phase liquid chromatography (RPLC) (Ultimate LC packings, Dionex) coupled on-line to a quadrupole-orthogonal-time-of-flight tandem mass spectrometer installed in the PI's lab(QSTAR XL, Applied Biosystems/MDS Sciex). RPLC was performed using a PepMap column (75 μm ID x 150 mm long, LC packings-Dionex) and the peptides were eluted using a linear gradient of 0% B to 35% B in 80 min at a flow of 200 nL/min. Solvent A contained 98% H_2O /2%acetonitrile/0.1% formic acid, whereas solvent B was composed of 98% acetonitrile /2% H_2O /0.1% formic acid. The QSTAR MS was operated in an information-dependent mode in which each full MS scan was followed by three MS/MS scans where the three most abundant peptide molecular ions were dynamically selected for collision induced dissociation (CID), thus generating tandem mass spectra. In general, the ions selected for CID were the most abundant in the MS spectrum, except that singly charged ions were excluded and dynamic exclusion was employed to prevent repetitive selection of the same ions within a preset time. Collision energies were programmed to be adjusted automatically according to the charge state and mass value of the precursor ions. To increase the number of MSMS spectra acquired from any given sample and improve the dynamic range of mass spectrometric analysis, multiple LC MS/MS runs were performed on the same sample with the exclusion lists (i.e. the m/z list of the ions being sequenced from the previous runs) generated from the previous LC MSMS runs using Mascot script within the Analyst program. The monoisotopic masses (m/z) of both parent ions and their corresponding fragment ions, parent ion charge states (z) and ion intensities from the tandem mass spectra (MS/MS) acquired was automatically extracted using the script in the Analyst software and directly submitted for automated database searching for protein identification using two different search engines, Protein Prospector (UC, San Francisco) and Mascot (Matrix Science), to improve confidence levels of the protein identifications in the large data sets. The LC-Batch tag program within the development version of

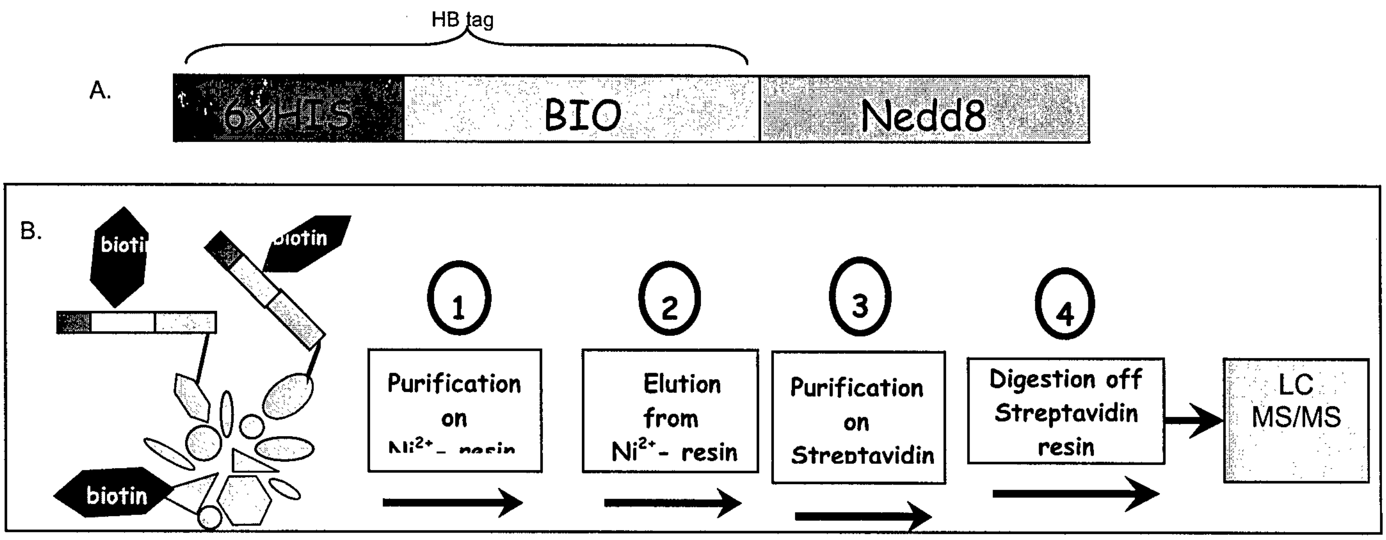

Fig. 2. (A) The new tandem affinity tag, HB tag consists of 6 histidine, a signaling peptide for biotintylation; (B) Tandem affinity purification strategy for HB-tagged Nedd8 modified proteins. The proteins bound on streptavidin were digested and analyzed by LC MS/MS analysis for protein identification.

Protein Prospector was used for database searching. The mass accuracy for parent ions and fragment ions were set as ±100 ppm and 300 ppm, respectively. An in-house mascot program was also used for the database searching and the mass accuracy for parent ions were set as ±100 ppm and 0.3 Da was used for the fragment ion mass tolerance. Both SwissProt and NCBInr public databases were queried to identify the purified proteins since each database contains unique protein entries. In addition, the Search Compare program within the development version of Protein Prospector {Chalkly, 2005} was used to make a list of proteins that differed between samples.

After mass spectrometric analysis and database searching, more than 100 proteins were identified in the FLAG elute. Among them, the most abundant proteins were heat shock proteins, however, none of the known substrates (e.g. cullin family members) were identified. Since affinity purification using FLAG tag can only be carried out in non-denaturing conditions, proteins due to non-covalent interactions and non-specific binding will be pulled out at the same time when nedd8 covalently modified proteins are purified. To eliminate the background due to non-covalent interaction as well as non-specific binding, we have adopted a newly developed tandem affinity purification tag, called HB tag, which has been developed to specifically purify covalently linked proteins such as ubiquitinated or sumoylated proteins by Prof. Peter Kaiser's lab at UCI. The HB-tag consists of a hexahistidine sequence combined with a signal peptide that serves as a biotinylation signal *in vivo* {Cronan, 1990}. HB-tagged proteins are efficiently biotinylated in vivo in yeast as well as in mammalian cells (>95%) at a specific lysine residue present in the tag. HB-tagged proteins can be purified by Ni^{2+}-chelate chromatography followed by binding to streptavidin resin under fully denaturing conditions as shown in Fig. 2. There is a small number (4-6) of endogenous biotinylated proteins present in eukaryotic cells. These proteins are effectively removed by the first purification step. The extraordinarily high affinity between biotin and streptavidin (Kd= 10^{-15} M) tolerates extremely stringent wash conditions such as 4% SDS and 8M urea (our observation) and thus guarantees highly purified samples. At the last step of the purification, the proteins bound on streptavidin resin can not be eluted; therefore on-beads digestion was performed prior to mass spectrometric analysis for protein identification as shown in Fig. 2B. To purify nedd8 modified proteins under fully denaturing conditions, the HB was tagged at the N-terminus of Nedd8, which was used for transiently transfection in Hela cells. Similar to what has been described above for 3XFLAG-Nedd8, experiments were carried out to optimize the protein expression and purification steps. Then western blotting using anti-RGSHis was performed to see whether any proteins were neddylated in the cells expressing HB-Nedd8 as shown in Fig. 3. Similar to Fig.1, western blotting has shown that there are many putative HB-Nedd8 modified proteins and the first step purification using Ni-NTA chromatography is quite efficient. To identify the putative Nedd8 modified proteins, the proteins bound on streptavidin were digested with trypsin overnight (Fig. 2B) and the digests were extracted and concentrated for LC MS/MS analysis. Based on peptide sequencing, about 50 proteins were identified, among which no cullin proteins were found either, and heat shock protein 70 was the most

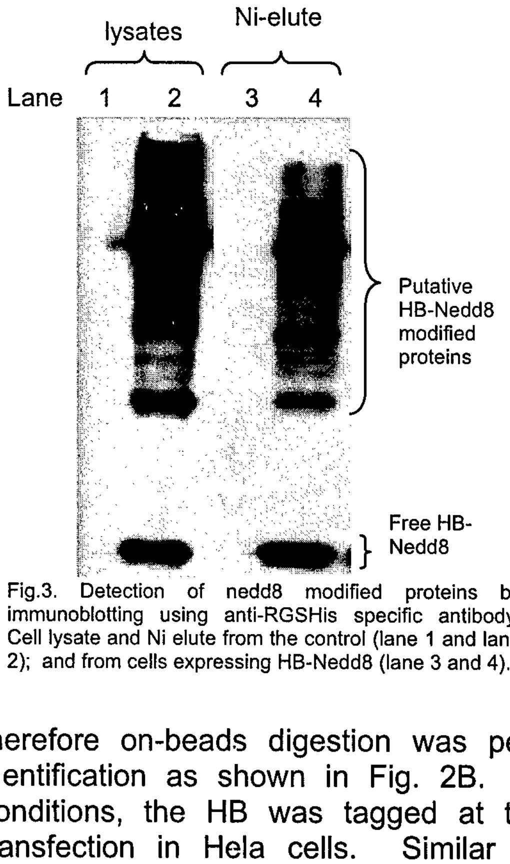

Fig.3. Detection of nedd8 modified proteins by immunoblotting using anti-RGSHis specific antibody. Cell lysate and Ni elute from the control (lane 1 and lane 2); and from cells expressing HB-Nedd8 (lane 3 and 4).

abundant protein in the purification, indicating overexpression of Nedd8 in cells may cause the stress to the cells. It is quite surprising to us that we were not able to identify the only known nedd8 substrates, i.e. cullin family members, even with the purification under fully denatured conditions. These results suggest that either Hela cells only contain very low amount of cullin members or overexpression of Nedd8 in cells by transient transfection might lead to unexpected outcomes.

To understand why no cullin proteins were identified, we have tried different experiments such as using another type of mammalian cell line, HEK 293 cell, for transient transfection purpose, and test the level of cul-1 in Hela and 293 cells using immunoblotting with anti-Cul-1 specific antibodies. When we tested the whole cell lysates from both Hela cells and 293 cells using immunoblotting with anti-cul-1 specific antibody, it is surprising that the level of Cul-1 in Hela cell is very low compared to that in 293 cells (data not shown). Therefore, we decided to use 293 cells for expressing tagged Nedd8. As shown in Fig.4, anti-Cul-1 immunoblotting has shown that Cul-1 was found as doublet in both non-transfected (control) and transfected 293 cells (lane 1 and 2). After FLAG affinity purification, only the eluents transfected cells (expressing 3xFLAG-Nedd8) has shown a band corresponding to Cul-1, not the control cells, indicating that Cul-1 was conjugated to p3XFLAG-Nedd8. Although Cul-1 was detected by western blot in 293 cells expressing p3XFALG-Nedd8, Cul-1 was not identified by mass spectrometric analysis when two of 150 mm plates of cells (~2 ml lysates) were used for affinity purification and subsequent LC MS/MS analysis. This suggests that the abundance level of p3XFLAG-Nedd8 conjugated Cul-1 from two 150 mm plates of cells is below the detection limit of our mass spectrometric analysis (~femtomole sensitivity). This was further confirmed when we co-overexpressed Cul-1 and p3XFLAG-Nedd8 in 293 cells, we were able to detect Cul-1 by LC MS/MS analysis.

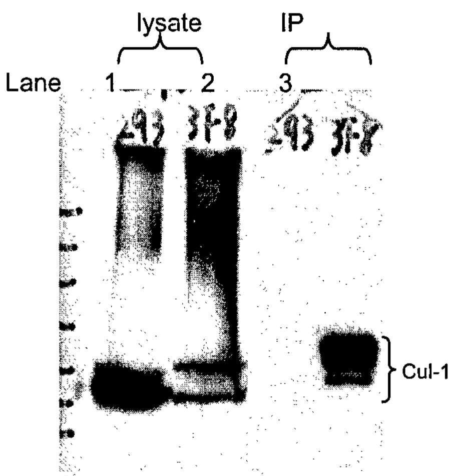

Fig. 4. Detection of Cul-1by immunoblotting with anti-Cul-1 antibody in HEK 293 cell lysates (lane 1: non-transfected; lane 2: transfected with p3XFLAG-Nedd8); and in immunoprecipitation eluents using anti-FLAG antibody M2 beads (lane 3: nontransfected cells; lane 4: cells transfected with p3X-FLAG-Nedd8). The doublet of Cul-1 indicates the part of the Cu-1 has been neddylated.

To solve the problem regarding the low level of nedd8 modified proteins and the effect of overexpression of Nedd8 on the cells, in June 2005, we decided to collaborate with Prof. Pan Zhen-Qiang at the Dept. of Oncological Sciences, Mt. Sinai School of Medicine, NY, who is an expert on E3 ubiquitin ligase especially Cullin containing E3 ubiquitin ligases. His lab has been studying the role of neddylation on the function of cullins, and has generated stable cell lines expressing FLAG-Nedd8, GST-Nedd8 and V5-Nedd8. Since the stable cell lines with GST-Nedd8 give best expression, we have started our analysis with this cell line. Stable cell line expressing GST-Nedd8 was grown in 150 x 25 mm tissue culture dishes until confluency. Two hundred and fifty plates of the cells were harvested and lysed as described {Wu, 2000}. 125 ml of lysates was incubated with 4 ml pre-equilibrated Glutathione Sepharose beads overnight at 4°C. The beads were then loaded into a chromatography column and washed with 100 ml lysis buffer followed by 100 ml buffer A50 (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% NP-40, 10 % glycerol, 50 mM NaCl. The bound proteins were then eluted with 100 ml buffer A50 plus 20 mM glutathione and concentrated 100 fold using 5000 MWCO Amicon Ultra centrifugal concentrators (Millipore). Approximately 2 mg of protein was obtained from 125 ml of lysates. About 15 μl of the eluent after concentration was loaded onto 1-D SDS PAGE and the protein bands were visualized by commassie blue staining as shown in Fig. 5A. In the molecular weight region of 75 ~200kDa, multiple bands were observed, corresponding well with the anti-Nedd8 western blot as shown in Fig. 5B. In addition to neddylated cullins, multiple other bands above 100kDa were also detected by anti-Nedd8 antibody, suggesting that other neddylated proteins may be present. To identify the GST-Nedd8 associated proteins, 18 bands between the molecular weight region of 75 kDa~200kDa were cut and in-gel digested by trypsin. The tryptic digests were extracted, concentrated and analyzed by LC MS/MS. More than 100 proteins were identified. Most excitingly, for the first time, we were able to identify all of the eight cullin-family members (i.e. Cul-1, Cul-2. Cul-3, Cul-4A, Cul-4B, Cul-5, Cul-7, Parc) in the GST-Nedd8 affinity purified proteins. As an example, Fig. 6 displays the MSMS spectrum of a tryptic peptide (MH_2^{2+} 590.24), the observance of the whole series of y ions has determined the peptide sequence as GMTENEVEDR, which matched to protein Cul-2 sequence unambiguously. Interestingly, the neddylation sites of all the cullin members have also been identified as illustrated in Fig. 7. The peptide sequence in Fig. 7 was determined as ImK(GG)mR, where m is oxidized methionine and K is covalently attached to two glycines (GG). The two glycine residues are the remnant of nedd8 after trypsin digestion, which is the characteristic of neddylation. This particular peptide sequence with nedd8 modification is conserved in Cul-1, Cul-4A/B and Cul-5. Similar, other nedd8 modified peptides were also identified for Cul-2 and Cul-3. Although neddylation of cullins has been revealed by immunoblotting and mutagenesis before, this is the first time that mass spectrometric analysis has been carried out and done successfully. Since the purification of GST-Nedd8 associated proteins was carried out under non-denaturing condition, in addition to nedd8 covalently modified proteins, their non-covalent protein interactions will be co-purified as well. During this prep, besides all cullin members, other components that form complexes with each cullin member have been co-purified and identified. Among the identified proteins, there are transcriptional factors, DNA repair complexes, DNA replication complexes, cell cycle regulatory complexes, etc. This is the first time that these complexes have been co-purified and identified during the purification of Nedd8 associated proteins. The results are not surprising since it is known that neddylation is important in cell development and embryogenesis. However, the molecular mechanisms of nedd8 pathway regulating these processes

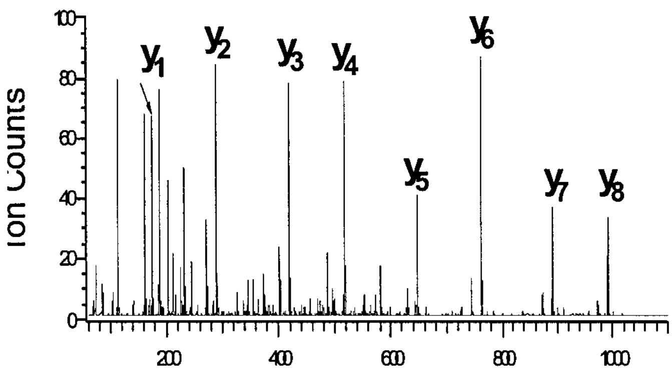

Fig. 6. MS/MS spectrum of a tryptic peptide(MH_2^{2+} 590.24) from band N1as shown in Fig. 5, which matched to Cul-2 unambiguously. The peptide sequence was determined as GMTENEVEDR.

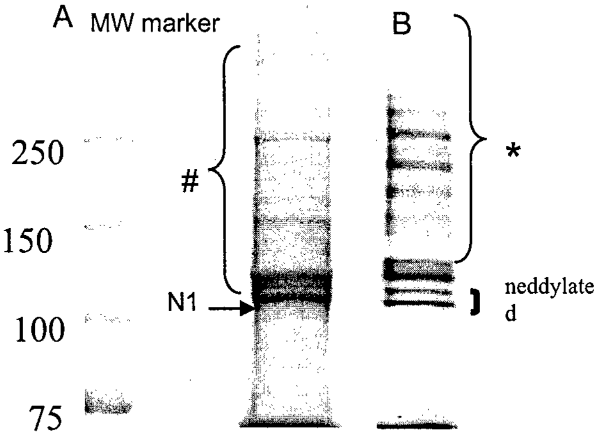

Fig. 5. (A) 1-D SDS gel electrophoresis picture of affinity purified GST-Nedd8 associated proteins from stable cell lines expressing GST-Nedd8 using Glutathione beads. (B) The immunoblot of the purified GST-Nedd8 associated proteins using anti-Nedd8 specific antibody. *: putative nedd8 modified substrates, #: the bands in this gel region has been cut and analyzed for protein identification.

will need to be further addressed. These results suggest that other neddylated substrates are probably present at very low level leading to the difficulty of detecting previously. Large preparation of these samples using more than 200 150mm plates actually made this analysis possible.

During this analysis, we have also identified components involved in proteasome-ubiquitin dependent degradation pathways such as proteasome 19S regulatory complex and ubiquitin itself. Further analysis is needed to understand the details of the molecular connections among neddylation, ubiquitination and degradation pathways. Similar experiments were also performed using stable cell lines expressing V5-Nedd8 and again the cullin family members were identified. The protein identification is quite reproducible among different preparations. Currently we are in the process of analyzing all the proteins purified including those located below 75kDa in the 1-D SDS-PAGE. Preliminary results have shown that nedd8 pathway may be involved in more different biological processes than we expected before.

In summary, we have finally obtained a stable cell line expressing GST-Nedd8 as our model system to study the nedd8 modified and associated proteins. This provides a necessary basis for us to further our analysis in prostate cells to understand the role of nedd8 pathway in cancer development.

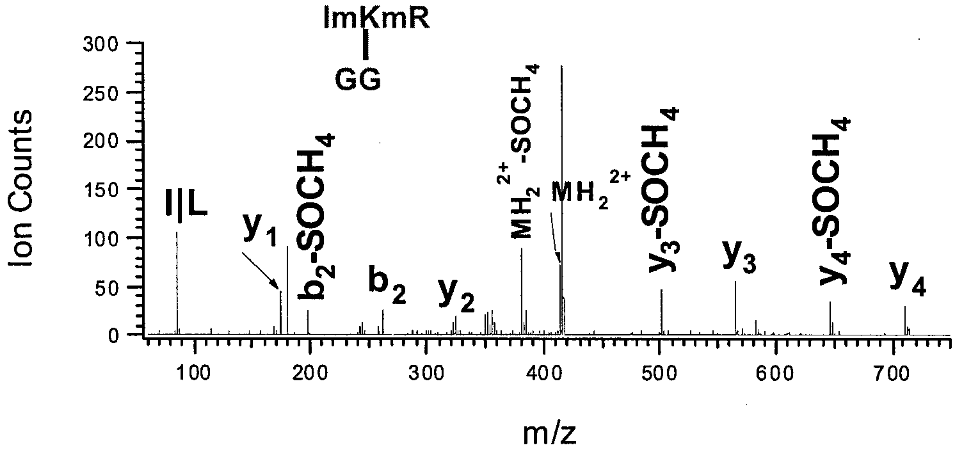

Fig. 7. MSMS spectrum of a nedd8 modified tryptic peptide (MH_2^{2+} 412.70) from band N2, which matched to protein Cul-1. The peptide sequence was determined as ImK(GG)mR, where the K was modified by GG.

c. Identify and quantify Nedd8 modified proteins using isotope-coded affinity tag technology (ICAT) and mass spectrometry.

This part of work will be initiated after we establish benign and malignant prostate epithelial cells (PrEC and LNCaP) expressing GST-Nedd8.

Key Research Accomplishments: During the first year of the funding period, we have tried really hard and done many experiments to fulfil task 2. Based on the work we have done on Hela and 293 cell lines, it seems that 293 cells are more suitable for transfection of tagged Nedd8 due to the abundance levels of endogenous cullin proteins. We have evaluated two different tags, e,g. 3xFLAG and HB tags, for nedd8 affinity purification. After extensive experiments, we have found that overexpression of Nedd8 in cells by transient transfection may lead stress to the cells since hsp70 has been the most abundant protein in the affinity purified fractions. However, transient transfection has been used for the study of ubiquitination and sumoylation. Therefore stable cell lines expressing tagged Nedd8 is preferred for our purposes. Another important thing we have found is that the neddylation is very dynamic process and the fraction of proteins being neddylated is low. Therefore, large quantity of cells (~10^8) are needed to affinity purify sufficient amount of Nedd8 modified and associated proteins for protein identification by mass spectrometry. In our recent preparation using a stable cell line expressing GST-Nedd8, we have not only identified all the cullin family members and their associated components, but also nedd8 modification sites on each cullin members. Among more than 100 proteins identified in the molecular region of 75 ~200kDa after 1-D SDS-PAGE (Fig. 5), there are transcriptional factors, DNA repair complexes, DNA replication complexes, cell cycle regulatory complexes, etc. This is the first time that these complexes have been co-purified and identified during the purification of Nedd8 associated proteins. It is noteworthy that mass spectrometric analysis has shown that nedd8 itself can be either neddylated or ubiquitinated which has not been reported before. It is well known that only ubiquitination can generate polyubiquitin chain in vivo as protein degradation signal, and ubiquitin-like proteins usually modify their substrates by addition of one ubiquitin-like protein, not forming polychain. If it is true that Nedd8 can form polyneddylation chain *in vivo*, that will be a very exciting discovery in this field. In summary, these results have established the solid foundation for us to fulfill the goals proposed in this work successfully in the future. In addition, the results may provide some new aspects regarding how nedd8 pathway affects cell cycle progression and development.

Reportable Outcomes : It is feasible to use different tags such as FLAG, GST, and V5 for affinity purification of tagged Nedd8 modified and asscoiated proteins. Stable cells expressing tagged Nedd8 is preferred for the study of the biological role of Nedd8 pathway in cellular development. Cullin family members are the only known and maybe the major substrates of neddylation. The neddylation sites of cullin family members revealed by mutagenesis study have been confirmed the first time by direct mass spectrometric analysis based on peptide sequencing.

Conclusions : We have established and ultilized a stable cell line system expressing tagged Nedd8 (e.g. GST, V5) to affinity purify nedd8 modified and associated proteins for subsequent mass spectrometric analysis. The identification of all cullin family members and their neddylation sites by mass spectrometry has provided the excellent fundation for the success of the proposed work in the remaining funding period.

References:

Chalkley RJ, Baker PR, Huang L, Hansen KC, Allen NP, Rexach M, Burlingame AL. Comprehensive analysis of a multidimensional liquid chromatography mass spectrometry dataset acquired on a quadrupole selecting, quadrupole collision cell, time-of-flight mass spectrometer: II. New developments in Protein Prospector allow for reliable and comprehensive automatic analysis of large datasets.

Wu K, Fuchs SY, Chen A, Tan P, Gomez C, Ronai Z, Pan ZQ, The SCF(HOS/beta-TRCP)-ROC1 E3 ubiquitin ligase utilizes two distinct domains within CUL1 for substrate targeting and ubiquitin ligation. Mol Cell Biol. 2000 Feb;20(4):1382-93.

Appendices : Our new collaborator, Prof. Pan Zhen-qiang's biosketch.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Pan, Zhen-Qiang	Associate Professor
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Fudan University, Shanghai, China	B.S.	1978-82	Microbiology
Columbia University, New York, NY	Ph.D	1983-89	Molecular Biology
Sloan Kettering Cancer Center, New York	Post-doctoral	1989-95	Biochemistry

Positions and Appointments:
1986-1989, Graduate Student (with Dr. Carol Prives), Columbia University, New York
1989-1995, Post-Doctoral Research Fellow (with Dr. Jerard Hurwitz), Sloan-Kettering, New York
1995-2001, Assistant Professor, Ruttenberg Cancer Center, Mt. Sinai School of Medicine, New York
2002-2003, Associate Professor, Ruttenberg Cancer Center, Mt. Sinai School of Medicine, New York
2004-present, Tenured Associate Professor, Dept. of Oncological Sciences, Mt. Sinai School of Medicine, NY

Honors and Awards:
1989, John Newberry Prize; 1991, Leukemia Society of America Fellowship Award; 1996, Life and Health Insurance Medical Research Fund Award; 1997, The New York Community Trust Cancer Research Award; 1998, Hirschl Career Scientist Award

Editorial Boards: 2004-2009, Journal of Biological Chemistry

NIH Study Section: Molecular Oncogenesis, 2005

A. **Selected peer-reviewed publications (in chronological order).** Do not include publications submitted or in preparation. **(Selected publications since 1996; out of a total of 54)**

1. Reardon, J.T.,Ge, H., Gibbs, E., Sancar, A., Hurwitz, J., and **Pan, Z.-Q.** (1996) Isolation and characterization of two human transcription factor IIH (TFIIH)-related complexes: ERCC2/CAK and TFIIH*. *Proc. Natl. Acad. Sci. USA* **93**, 6482-6487
2. **Pan, Z.-Q.**, Ge, H., Amin, A., and Hurwitz, J. (1996) Transcription positive cofactor 4 forms complexes with HSSB (RPA) on single-stranded DNA and influences HSSB-dependent enzymatic synthesis of simian virus 40 DNA. *J. Biol. Chem.* **271**, 22111-22116
3. Ko, L.J., Chen, X., Shieh, S.-Y., Layaraman, L., Taya, Y., Prives, C and **Pan, Z.-Q.** (1997) p53 is phosphorylated by CDK7/cyclin H In p36/MAT1 dependent manner. *Mol. Cell. Biol.* **17**, 7220-7229
4. Fuchs, S.Y., Chen*, A., Xiong, Y., **Pan, Z.-Q.**, and Ronai, Z. (1999) HOS, a human homologue of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkB and b-catenin. *Oncogene* **18**, 2039-2046
5. Tan, P., Fuchs, S.Y., Chen, A., Wu, K., Gomez, C., Ronai, Z., and **Pan, Z.-Q.** (1999) Recruitment of a ROC1:Cullin1 ubiquitin ligase by Skp1 and HOS to catalyze theubiquitination of IkBa. *Mol. Cell* **3**, 527-533
6. Santagata, S, Besmer, E., Villa, A., Bozzi, F., Allingham, J.S., Sobacchi, C., Haniford, D.B., Vezzoni, P., Nussenzweig, M.C., **Pan, Z.-Q.**, and Cortes, P. (1999) The RAG1/RAG2 complex constitutes 3' flap

endonuclease activity: implications for junctional diversity in V(D)J and transpositional recombination. *Mol. Cell* 4, 935-947

7. Wu, K., Fuchs, S.Y., Chen, A., Tan, P., Gomez, C., Ronai, Z., and **Pan, Z.-Q.** (2000) The SCF$^{HOS/\beta\text{-}TRCP}$-ROC1 E3 Ubiquitin Ligase Utilizes Two Distinct Domains within CUL1 for Substrate Targeting and Ubiquitin Ligation. *Mol. Cell. Biol.* **20**, 1382-1393
8. Chen, A., Wu, K., Fuchs, S.Y., Tan, P., Gomez, C., and **Pan, Z.-Q.** (2000) The conserved RING-H2 finger of ROC1 is required for ubiquitin ligation. *J. Biol. Chem.* **275,** 15432-15439
9. Wu, K, Chen, A, and **Pan, Z.-Q.** (2000) Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. *J Biol Chem.* **275,** 32317-32324
10. Kentsis A, Dwyer EC, Perez JM, Sharma M, Chen A, **Pan, Z.-Q,** Borden KL. (2001) The RING domains of the promyelocytic leukemia protein PML and the arenaviral protein Z repress translation by directly inhibiting translation initiation factor eIF4E. *J Mol Biol.* **312(4):** 609-23
11. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, **Pan, Z.-Q,** Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294(5547):** 1704-8
12. Wu K, Chen A, Tan P, **Pan, Z.-Q.** (2002) The Nedd8-conjugated ROC1-CUL1 Core Ubiquitin Ligase Utilizes Nedd8 Charged Surface Residues For Efficient Polyubiquitin Chain Assembly Catalyzed By Cdc34. *J Biol Chem* **277:** 516-527.
13. Chen A, Kleiman FE, Manley JL, Ouchi T, **Pan Z-Q.** (2002) Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase. *J Biol Chem* **277(24):** 22085-92.
14. Kim J, Kim JH, Lee SH, Kim DH, Kang HY, Bae SH, **Pan Z-Q**, Seo YS. (2002) The novel human DNA helicase hFBH1 is an F-box protein. *J Biol Chem.* **277(27):** 24530-7.
15. Macip S, Igarashi M, Fang L, Chen A, **Pan Z-Q**, Lee SW, Aaronson SA. (2002) Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *EMBO J.* **21(9):** 2180-8.
16. Dias, DC, Dolios, G, Wang, R., **Pan, Z-Q**. (2002) CUL7: a DOC domain-containing cullin selectively binds Skp1•Fbx29 to form a novel SCF-like complex. *Proc. Natl. Acad. Sci. USA*, 99(26): 16601-6
17. Wu K, Yamoah K, Dolios G, Gan-Erdene T, Tan P, Chen A, Lee CG, Wei N, Wilkinson KD, Wang R, Pan ZQ. (2003) DEN1 is a dual function protease capable of processing the C-terminus of Nedd8 deconjugating hyper-neddylated CUL1. *J. Biol. Chem.,* **278:** 28882-91.
18. Gan-Erdene T, Kolli N, Yin L, Wu K, Pan ZQ, Wilkinson KD. (2003) Identification and characterization of DEN1, a deneddylase of the ULP family. *J. Biol. Chem.,* **278:** 28892-900.
19. Pan, Z.-Q., Kentsis, A, Dias, DC, Yamoah, K, and Wu, K. (2004) Nedd8 on Cullin: Building an Expressway to Protein Destruction. *Oncogene*, **23:** 1985-1997.
20. Li, Y., Gazdoiu, S., Pan, Z.-Q., and Fuchs S.Y.. (2004) Stability of homologue of Slimb F-box protein is regulated by availability of its substrate. *J. Biol. Chem.,* **279:** 11074-80.
21. Reverter D, Wu K, Erdene TG, Pan ZQ, Wilkinson KD, Lima CD. (2005) Structure of a complex between Nedd8 and the Ulp/Senp protease family member Den1. *J Mol Biol.* **345(1):** 141-51
22. Miller F, Kentsis A, Osman R, and Pan ZQ. (2005) Inactivation of VHL by Tumorigenic Mutations that Disrupt Dynamic Coupling of the pVHL:HIF-1α Complex. *J. Biol. Chem.* **280:** 7985-7996
23. Yamoah, K., Wu, K. and Pan, Z.-Q. (2005) In vitro cleavage of Nedd8 from cullin 1 by COP9 Signalosome & Deneddylase 1. *Methods Enzymology*, **398:** 509-522.
24. Huber C, Dias-Santagata D, Glaser A, O'Sullivan J, Brauner R, Wu K, Pearce K, Wang R, Luisa M, Uzielli G, Dagoneau N, Chemaitilly W, Superti-Furga A, Dos Santos H, Mégarbané A, Morin G, Gillessen-Kaesbach G, Hennekam R, Brunner H, Graeme Black GCM, Clayton PE, Read A, Le Merrer M, Scambler PJ, Munnich A, Pan ZQ, Winter R, and Cormier-Daire V. (2005) Identification of CUL7 mutations in the 3-M syndrome. *Nature Genetics*, **37:** 1119-1124.
25. Gazdoiu, S, Yamoah, K, Wu, K, Escalante, CR, Tappin, I, Bermudez, V, Aggarwal, AK, Hurwitz, J, and Pan, ZQ. (2005) Proximity-induced activation of human Cdc34 through heterologous dimerization, *Proc. Natl. Acad. Sci. USA*, **102:** 15053-15058.

B. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

Ongoing Research Support

1. RO1 GM61051, Pan 01/01/02-12/31/05
   National Institutes of Health
   Studies on ROC1-dependent ubiquitin E3 ligases
   The major goals of this proposal are to: 1) determine the reaction mechanism of ROC1-Mediated ubiquitin ligation; 2) investigate the role of Nedd8 modification in activating the ubiquitin ligase activity of the ROC1-CUL1 complex; and 3) examine the role of Fbx22^{p44} in targeting the KLF6 transcription factor for ubiquitination.
   Role: PI

2. RO1 CA095634, Pan 07/01/03-06/30/07
   National Institutes of Health
   The function and regulation of the cullin family E3 ubiquitin ligases
   The major goals of this proposal are to: 1) determine the biological function of the CUL7/Fbx29-based E3-like complex; 2) determine the regulatory role of DEN-1in the Nedd8 pathway; and 3) investigate mechanisms that regulate the pVHL-mediated ubiquitination of HIF-1α by Nedd8 and prolyl hydroxylase.
   Role: PI

Completed Research Support

1. R29 GM55059, Pan (PI) 08/01/97-07/31/02
   National Institutes of Health
   Mechanism and control of human nucleotide excision repair
   The major goals of this proposal are to understand: 1) the role of TFIIH in nucleotide excision repair particularly during damage recognition and coordination of repair and cell cycle checkpoint controls; and 2) the mechanism coupling post-incision and gap-filling DNA synthesis reactions.
   Role: PI

2.*RO1 AI 40191 04/01/97-03/31/01
National Institutes of Health
Biochemistry and regulation of V(D)J recombination

* This grant was originally awarded to Dr. Eugenia S. Spanopoulou, who perished in the Swiss Air crash of late 1998. At the request of Mount School of medicine, it was transferred to Dr. Pan, who has been taking up the leadership role to assure the successful completion of the projects outlined in the original proposal.